Translocases: **A bacterial tunnel for drugs and proteins**

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Unrelated translocases extrude proteins or antimicrobial agents across both membranes of the cell envelope in Gram-negative bacteria. The TolC protein links the translocases to the external environment. The recently determined crystal structure of TolC shows how this universal tunnel operates.

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When the bacterium *Escherichia coli* needs to move things out of the cell, whether it is a protein toxin aimed at the host or an unwanted antibiotic molecule that leaked in, the outer-membrane protein TolC is often the final portal in the pathway of transport. A recent paper by Koronakis and co-authors [1] reports the crystal structure of TolC at 2.1 Å resolution (Figure 1) and suggests how this universal tunnel operates.

Some pathogenic strains of *E. coli* that cause urinary tract infections carry plasmids that code for α-hemolysin, a protein toxin that inserts into the membrane of host cells, such as erythrocytes, makes a pore and causes them to lyse. Apparently, a weakened host makes a better medium in which *E. coli* can propagate. Apart from α-hemolysin, the plasmid codes for the toxin transporter, which is composed of two different parts. One is a translocase proper, HlyB, a member of the 'ATP-binding cassette' (ABC) transporter family. The other is a 'membrane fusion' protein, HlyD. A continuous channel forms, apparently by HlyD docking to the outer-membrane protein TolC. The complex spans two membranes and the periplasm [2] (Figure 2).

TolC is made of three monomers, which is typical for many outer membrane porins that form narrow channels allowing the passage of nutrients into the cell. Like the porins, the outermost part of TolC that is embedded in the lipid bilayer of the outer membrane forms a β-barrel structure, with hydrophobic side chains pointing to the lipid. Unlike porins, where the narrow channel is formed by a β-barrel of each monomer [3], each TolC peptide makes one-third of the wall of a very large, 35 Å inner diameter cylindrical tunnel. The β-barrel sheet of each monomer is adjacent to its neighbors, so a continuous cylinder is formed. The structure of TolC leaves little doubt about its function — this is a tunnel through which very large molecules can move.

Bridging the periplasm

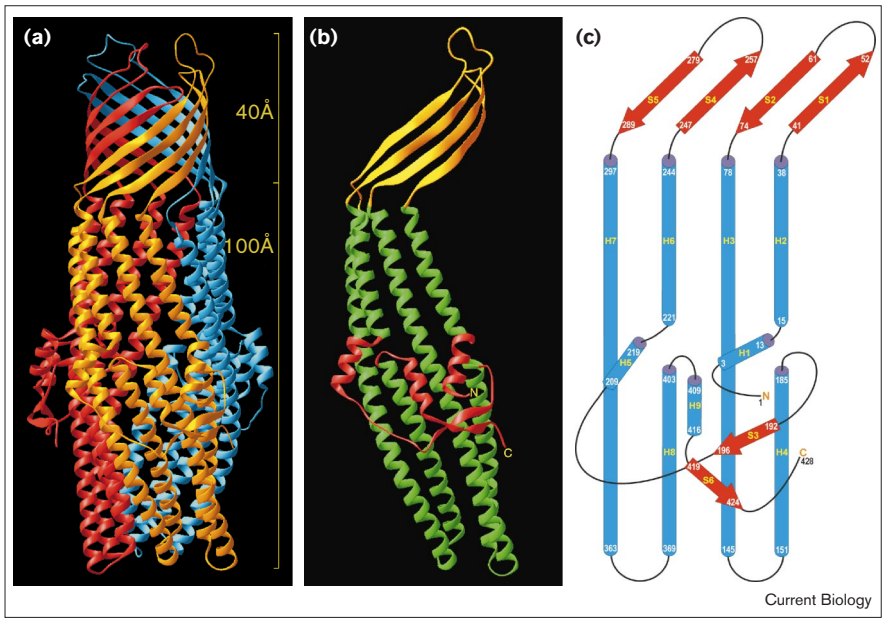
After crossing the outer membrane, the TolC cylinder extends for a further 100 Å into the periplasm. This part of the structure is geometrically similar to the β-barrel, with the three monomers forming adjacent sheets, but here of α-helices, that make up the wall of the tunnel. This novel protein fold has been named 'α-helical barrel'. The chemistry of the periplasmic domain of the molecule is very different from the outer-membrane part — the hydrophilic side chains of the α-helices extend into the periplasm, and into the lumen of the tunnel. Both the dimensions and the polarity of the α-helical part of the protein clearly indicate that it resides in an aqueous environment of the periplasm.

The presence of a large hydrophilic portion of the TolC tunnel is a fairly unanticipated finding. The role of crossing the periplasm has been so far assigned to the membrane fusion proteins, such as HlyD [4,5]. The structure of HlyD is not known, but sedimentation and light-scattering studies of the homologous AcrA component of a multidrug pump that also docks to TolC suggest a highly elongated structure with an 8:1 axial ratio [5]. The protein family that HlyD and AcrA belong to also includes simian virus 5, a distant, but distinct relative [6]. This viral protein has a true membrane-fusion function, fusing adjacent host cells and allowing the virus to expand.

All indirect evidence pointed to a minimal model in which TolC was restricted to the outer membrane (like other porins), while the elongated membrane fusion protein linked the membranes (like the simian virus fusion protein). Now direct evidence shows that this model needs revision, and the bridge that spans the periplasm is likely made of two parts — TolC and a membrane fusion protein. To be precise, the fusion protein is anchored in the inner membrane, but it actually brings together not membranes, but proteins — TolC and an inner membrane translocase. But why is HlyD homologous to the real membrane fusion protein of simian virus?

If α-hemolysin passes into TolC from the membrane fusion protein, one can expect that a continuous tunnel is made by the HlyD–TolC complex. Interestingly, HlyD forms a trimer [2], which would suggest a seamless link between the two parts of the tunnel. The dimensions of a possible HlyD tunnel structure can be estimated on the bases of what we know about TolC. The design of TolC is very economical — almost the entire mass of the trimer is distributed in a sheath that makes up the wall of the tunnel. The TolC trimer is made of 1413 amino acids that

Figure 1



The architecture of TolC. **(a)** Structure of the TolC trimer. Each subunit is shown in a different color. The subunits are adjacent to each other, and the overall structure forms a tunnel. The upper part, 40 Å long, is made of a β-barrel structure and is located in the outer membrane. The larger lower part, 100 Å long, is made of α-helices. **(b)** A single subunit of TolC. **(c)** The topology diagram of a TolC subunit. A structural repeat coincides with a repeat in the polypeptide sequence – apparently the result of gene duplication – and comprises segments (H1, H2, S1, S2, H3, H4) and (H5, H6, S4, S5, H7, H8). (Reproduced, with permission, from [1].)

form a cylinder 140 Å long and about 35 Å in internal diameter. Given the same length:width:amino acids ratio, a HlyD trimer of 1437 amino acids has a mass very similar to the TolC trimer, and could form a cylinder 35 Å in diameter that is about 140 Å long. This would provide a very long, 240 Å structure to bridge the periplasm. A crystal structure of a fusion protein, and of its complex with TolC, will show whether such a model is realistic.

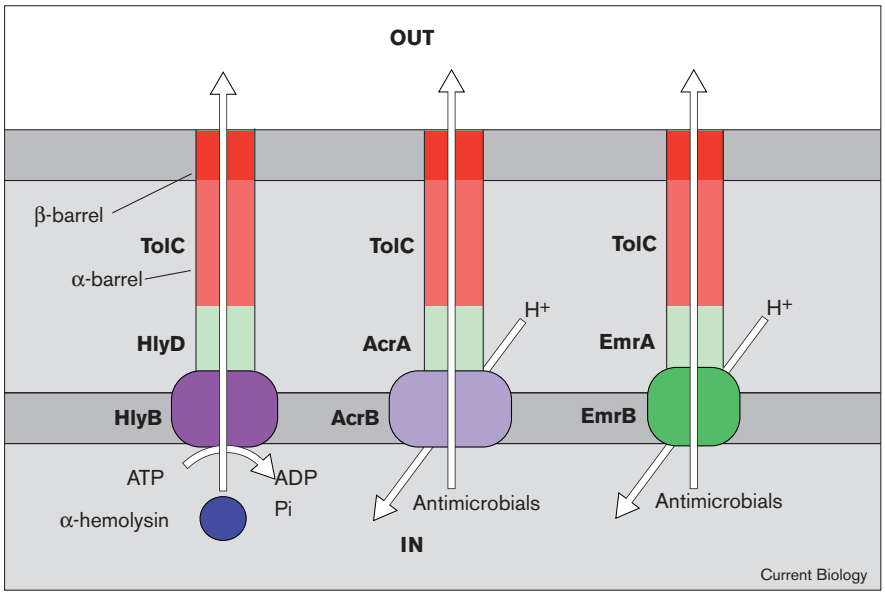
Given that TolC and a membrane fusion protein need to bridge the periplasm, it would be nice to know exactly how wide the space is. There is no consensus on this seemingly simple matter, perhaps reflecting the shifting nature of the periplasm. Changes in osmolarity cause expansion/contraction of the periplasm, so its width will depend on external conditions. Zones of adhesion, where the outer and inner membrane form a close contact, have been reported, and it is possible that proteins such as HlyB–HlyD–TolC are located in such zones. Might envelope spanning proteins like HlyB–HlyD–TolC actually form adhesion zones, and help keep the membranes together?

The α-hemolysin translocase is only made in some pathogenic strains of *E. coli*, so this particular translocase cannot be essential for adhesion zone formation. Besides, the HlyB–HlyD–TolC complex is a temporary structure; only HlyB–HlyD that is bound to α-hemolysin can dock to TolC [2]. In the absence of α-hemolysin, TolC is free to associate with other translocases, like the multidrug resistance proteins. Multidrug resistance proteins might form a more permanent connection to TolC — these proteins are encoded by chromosomal genes and are present in all strains of *E. coli*. In model experiments, an AcrAB complex was capable of transporting ligands across the membrane of a proteoliposome [7]. This would suggest that, *in vivo*, a more permanent AcrAB–TolC complex ensures that toxic ligands will not leak into the periplasm.

Whether adhesion zones are permanent structures of the cell envelope or not, a membrane-bridging location will subject TolC to stress — variations in osmolarity of the environment cause the periplasm to expand and contract. Interestingly, the entire structure of TolC [1] looks like a combination of two relaxed springs linked by a flexible joint. The α-helical strands wind around the sides of the tunnel, and prolines at the junction of the periplasmic and membrane domains allow for a tilt in the β-barrel in the opposite direction, forming an angled joint. The α-helical strands are supercoiled. These different levels of flexibility might allow the protein to stretch, decreasing the danger of the complex coming apart.

There is another interesting feature of the protein that might have evolved in response to the shifting environment of the periplasm. The end of TolC pointing towards the periplasm is closed. Koranakis *et al.* [1] suggest that, upon interaction with the translocase complex, the coils closing TolC unwind, forming a large opening into the tunnel. But why should there be a gate in the first place? If an expansion of the periplasm tears the complex apart, an open-ended TolC would make a huge hole in the outer membrane. This could lead to a rapid penetration of toxins, including small proteins such as lysozyme, into the periplasm. But the moment TolC disengages from a

Figure 2



TolC as a universal tunnel. Three different *E. coli* translocases use homologous 'membrane fusion proteins' to dock to TolC. This design allows for a transport of molecules across the entire cell envelope. HlyB is the translocase of α-hemolysin that is expressed in some pathogenic strains of *E. coli*. HlyB is an ABC-family translocase. HlyD is the membrane fusion protein. AcrB belongs to the RND family and is a multidrug resistance pump that extrudes a wide variety of antimicrobials, such as β-lactams, fluoroquinolones, antiseptics and detergents. The actual point of entry of AcrB substrates might be the outer bilayer of the inner membrane. EmrB is a 'major facilitator' family transporter that extrudes uncouplers of oxidative phosphorylation, a hydrophobic antibiotic thiolactomycin and nalidixic acid.

membrane fusion protein, the gate will close, preventing the formation of a dangerous leak.

Evacuating toxic drugs

Why does TolC serve as a channel for both protein toxins and toxic drugs? Structural biology is much better suited to addressing the question of 'how' than 'why'. Indeed, the structure [1] clearly shows that TolC is designed to accommodate the transport of small molecules as well as proteins. The coiled coils of the α-helices are kept together by a 'knobs-into-holes' packing of amino-acid side chains. In this arrangement, small aliphatic side chains from one helix (knobs) fit into a ring (hole) of four hydrophobic side chains of the neighboring helix. This arrangement literally plugs all the holes in the TolC tunnel through which small molecules might leak. This ensures that the toxins extruded by the multidrug pumps do not leak into the periplasm.

One-step transport of antimicrobial agents across the entire cell envelope provides Gram-negative bacteria with an enormous drug-resistance capability. Our studies in search of a mechanism of *E. coli* resistance to agents that uncouple oxidative phosphorylation indicated, unexpectedly, that the responsible protein is a membrane translocase [8]. The protein, EmrB, is homologous to other translocases of the 'major facilitator' family of proteins, which reside in the inner membrane. This finding was puzzling, as the very mechanism of uncoupling by compounds such as CCCP relies on their ability to shuttle back and forth across the inner membrane carrying protons. One might expect that a translocase 'extruding' CCCP across the inner membrane would only increase its toxic activity!

We found that a second protein, EmrA, was encoded by the same operon as EmrB, and EmrA showed sequence homology to HlyD. We suggested [8], by analogy to HlyB–HlyD–TolC, that EmrA–EmrB transports drugs all the way across the entire cell envelope. This would solve the apparent 'uncoupler extrusion' paradox, as the outer membrane, unlike the cytoplasmic membrane, is a fairly good barrier for amphipathic molecules such as CCCP and other toxins. It was subsequently shown that unrelated multidrug resistance proteins, such as *E. coli* AcrAB, are also associated with a membrane fusion protein homolog of HlyD, and 'take advantage' of the good barrier functions of the outer membrane [9].

Variations in extrusion design

In *E. coli*, unrelated HlyB, EmrB and AcrB are all associated with homologous membrane fusion proteins, which act as adaptors that dock these translocases to the same TolC port. But this arrangement might not be typical — in *Pseudomonas aeruginosa*, for example, operons encoding homologs of both AcrAB and EmrAB also carry genes for TolC homologs, so these systems do not rely on a universal outer-membrane portal. It will be interesting to learn whether these *Pseudomonas* outer-membrane proteins form wide tunnels, such as TolC, or rather make narrow channels, suited only for the passage of drugs. A different example of a narrower function is provided by a plant pathogen *Erwinia chrysanthemi*, which uses Prt proteins related to HlyBD to transport proteases from the cell, and the translocase operon also codes for a TolC homolog [10]. In this case, one would expect a tunnel structure for the TolC homolog to transport the large proteases, and perhaps a looser wall, not necessarily tightly plugged against leakage of small molecules.

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